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In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by *Helichrysum caespititium*

JJM Meyer*, N Lall and ADM Mathekga

Department of Botany, University of Pretoria, Pretoria 0002, South Africa
* *Corresponding author, e-mail: marion@scientia.up.ac.za*

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Antimycobacterial activity of acetone and water extracts of *Helichrysum caespititium* against a drug-sensitive strain of *Mycobacterium tuberculosis* was evaluated by the agar plate method. The acetone extract exhibited inhibitory activity at a concentration of 0.5mg ml^{-1} against this strain whereas the organism was found to be partially susceptible to the water extract at 5.0mg ml^{-1}. The inhibitory activity of the acetone extract was confirmed using the rapid radiometric method and the minimum inhibitory concentration (MIC) was found to be 0.1mg ml^{-1}. Caespitate, a novel phloroglucinol, which was previously isolated and identified, from *H. caespititium* was also evaluated for its activity against drug-sensitive and drug-resistant strains of *M. tuberculosis*. The MIC of caespitate was found to be 0.1mg/ml for all the *M. tuberculosis* strains. The ability of caespitate to inhibit the growth of all the strains of *M. tuberculosis*, shows the broad spectrum antimycobacterial activity of the compound.

Introduction

The resurgence of tuberculosis (TB) in recent years in many regions of the world, especially in developing nations has made the search for novel strategies to combat this infectious disease indispensable. TB is the leading cause of death worldwide claiming more adult lives than diseases such as AIDS, malaria, diarrhoea, leprosy and all other tropical diseases combined (Zumla and Grange 1998). About one third of the world's population is currently infected with *Mycobacterium tuberculosis,* while 10% of those infected will develop the clinical disease, particularly those with human immunodeficiency virus (HIV) infection (Wilkinson *et al*. 1996). According to World Health Organisation it is estimated that between 2000 and 2020, nearly 1 billion more people will be newly infected, 200 million people will get sick and 70 million will die from TB if the control of the disease is not strengthened (New Scientist 1998).

The organism usually responsible for TB is the tubercle bacillus, *Mycobacterium tuberculosis,* discovered by Robert Koch in 1882. TB is usually acquired by inhalation of the bacillus from an infectious patient and causes irreversible lung destruction. Today, many bactericidal and bacteriostatic drugs are used in combination all over the world with dramatic positive results and tuberculosis has become a curable disease. Even with the advent of such powerful specific antimicrobials, a long duration of treatment with either daily or intermittent therapy is required. In the presently available treatment regimes of 6–9 months duration, the patients frequently stop taking drugs as soon as the symptoms are ameliorated and the treatment is therefore discontinued. This leads to the emergence of multidrug resistant TB, a problem for which more toxic second-line drugs, have to be used. According to a WHO report, globally, 2% of all cases of tuberculosis are multidrug resistant — by definition, resistance to rifampicin plus isoniazid (plus other resistances). Such cases can be treated in the USA and other high - resource regions but at a great cost (>US$250 000 per case!) and using very long courses of rather toxic drugs, thereby raising serious problems of compliance (WHO 1997). South Africa is witnessing an explosion in the number of cases of drug-resistant tuberculosis. An estimated 2 000 South Africans contract multidrug resistant TB each year and more than half of these patients die within a period of two years (WHO/TB/98.258 1998). It is essential to have new antituberculosis agents, preferably those that can readily and simply be produced from some local source.

Plants have been used worldwide in traditional medicines for the treatment of various diseases. It is estimated that even today, approximately two-thirds to three-quarters of the world's population rely on medicinal plants as their primary source of medicines (Phillipson and Anderson 1989). The use of some medicinal plants such as *Vernonia woodii, Conyza ivaefolia,* a number of *Helichrysum* species such as *Helichrysum caespititium, H. leiopodium, H. appendiculatum,* etc., by South Africans in curing TB-related symptoms such as cough, fever, blood in the sputum has been reported. The leaves of *H. imbricatum* and *H. nudifolium* are being used as a tea and an infusion as a demulcent in coughs and in pulmonary affections. It has been reported that people smoke

the dried flower and seed of *H. krausii* in a pipe for the relief of cough and as a remedy for pulmonary tuberculosis (Watt and Breyer-Brandwijk 1962). However, only a small proportion of plant species have been thoroughly investigated for their medicinal properties and undoubtedly there are many novel biologically active compounds to be discovered.

Helichrysum caespititium (DC.) Harv. (Asteraceae) is a prostrate, perennial, mat-forming herb that is profusely branched and densely tufted. It has been reported that the Basotho, a South African tribe, and indigenous people of Zimbabwe inhale smoke from the burning plant for the relief of headache, colds and chest pain. Exudates of this herb are claimed to be effective against broncho-pneumonial diseases, sexually transmitted diseases, tuberculosis and ulceration (Watt and Breyer-Brandwijk 1962, Gelfand *et al*. 1985). However, not much attention has been given to the laboratory evaluation and detection of antituberculosis activity of *H. caespetitium*. We, therefore, decided to investigate the antimycobacterial effect of plant extracts and a phloglucinol isolated from it against drug-sensitive and drug-resistant strains of *M. tuberculosis*. We also compared our results of susceptibility testing by the agar plate method with that of the radiometric method.

Materials and Methods

Plant material

Shoots of *H. caespititium* were collected near Harrismith and identified at the HGWJ Schweickerdt herbarium of the University of Pretoria and also at the herbarium of the National Botanical Institute, Pretoria. A voucher specimen (AM11) of the species was deposited in the herbarium of the National Botanical Institute of South Africa, in Pretoria.

Preparation of plant extracts

Dried aerial plant parts of *H. caespititium* (40g) were immersed in acetone and shaken on a rotary shaker for 5 minutes. The extract was filtered and evaporated to dryness in vacuo at 40°C. The acetone residue was dissolved in dimethyl sulphoxide (DMSO) to obtain a final concentration of 500mg ml^{-1} because of the toxicity of acetone towards bacteria.

The water extract was prepared by boiling ca. 20g of plant material in 500ml of distilled water for 30min under reflux. The extract was filtered and concentrated to dryness at reduced pressure. The residue was dissolved in distilled water to give a final concentration of 500mg ml^{-1}.

Isolation of a phloroglucinol

A novel phloroglucinol, caespitate (2-methyl-4-[2',4',6'-trihydroxy-3'-(2-methylpropanoyl) phenyl]but-2-enyl acetate) [1] was isolated from the crude acetone extract of *H. caespititium* (Figure 1) as described earlier by Mathekga *et al.* (2000). The crude acetone extract was initially subjected to preparative thin layer chromatography in $CHCl_3$-EtOAc (1:1). The targeted band was recovered and rechromatographed by column chromatography with 100% chloroform on silica gel 60. The compound was finally purified by high performance liquid chromatography in H_2O-Et OH (1:1) on a reverse phase Phenomenex column (250 x 4.60mm, 5m).

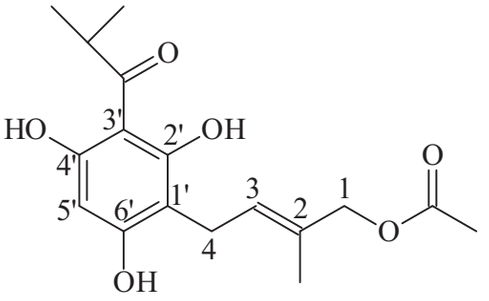

Figure 1: Caespitate (2-methyl-4-[2',4',6'-trihydroxy-3'-(2-methylpropanoyl) phenyl]but-2-enyl acetate) isolated from *Helichrysum caespititium*

Determination of antimycobacterial activity

Preparation of bacterial media

One litre of Middlebrook 7H11 agar (Difco Laboratories) containing 0.5% glycerol was prepared for susceptibility testing by the agar plate method (Middlebrook and Cohn 1958). The acetone and water plant extracts (500mg ml^{-1}) were sterilised by filtration through 0.22mm syringe fitted filters and then incorporated in the medium before solidification, to obtain final concentrations of 5.0, 1.0 and 0.5mg ml^{-1}. Control experiments showed that the final amount of DMSO (1%) in the media had no effect on the growth of *M. tuberculosis.* The mixture (10ml) of plant extract and medium was poured in glass bottles and solidified in slants. All tests were done in triplicate.

Preparation of inocula and interpretation of results

Bacterial cultures utilised in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. These cultures were routinely tested for susceptibility to the primary drugs streptomycin (SM), isoniazid (INH), ethambutol (EB) and rifampicin (RIF). A drug-sensitive strain of *M. tuberculosis*, H37Rv reference strain, was also used in the screening procedure.

Standard inoculum was prepared for the sensitive strain in Middlebrook-Dubos 7H9 broth containing 0.5% Tween 80 to obtain a concentration of 1mg ml^{-1} (wet mass) as described previously by Lall and Meyer (1999). The H37Rv sensitive strain suspension was divided into two portions. One portion was saved for the rapid radiometric susceptibility test and the other was used for the agar plate susceptibility testing.

The suspension was diluted to $1x10^{-2}$mg ml^{-1} and $1x10^{-4}$ mg ml^{-1}. To each bottle containing plant extract, 0.2 ml of the $1x10^{-2}$mg ml^{-1} of bacterial suspension was added. For the control tubes (medium + 1% DMSO), 0.2ml of the two dilutions ($1x10^{-2}$ and $1x10^{-4}$mg ml^{-1}) of the inoculum were used.

The antimicrobial activity was evaluated after 6 weeks of incubation at 37°C. The number of colonies growing on the

medium with plant extracts, containing the dilution $1x10^{-2}$mg ml^{-1} of the inoculum (N^{-2}) was compared with the growth on the control series, containing dilutions $1x10^{-2}$mg ml^{-1} (NO^{-2}) and $1x10^{-4}$mg ml^{-1} (NO^{-4}) of the inoculum.

The following criteria were used for the interpretation of the results:

$N^{-2} \geq NO^{-2}$: the strain is considered as resistant;

$NO^{-4} \leq N^{-2} \leq NO^{-2}$: the strain is considered as partially susceptible;

$N^{-2} \leq NO^{-4}$: the strain is considered as sensitive (<1% growth).

All procedures involving transfer of cultures were carried out in a biological safety cabinet.

The radiometric method

The activity of acetone plant extract was found to be 0.5mg ml^{-1} against the H37Rv strain by the agar plate method. It was decided to compare this result with the radiometric method. Acetone extract of the plant was analysed for activity at concentrations of 1.0, 0.5 and 0.1mg ml^{-1} against a sensitive strain. A novel phloroglucinol, caespitate (2-methyl-4-[2',4',6'-trihydroxy-3'-(2-methylpropanoyl) phenyl]but-2-enyl acetate) [1] isolated from the plant was also evaluated against drug-sensitive and 2–7 drug resistant strains at concentrations of 0.1, 0.05 and 0.01mg ml^{-1} (Table 1). All tests were done in triplicate.

The crude acetone extract and the compound [1] were each dissolved at 10mg ml^{-1} in 1% DMSO and stored at -4°C until used. Subsequent dilutions were done in DMSO and added to 4ml of BACTEC 12B broth (7H12 medium) to achieve the desired final concentrations together with PANTA (Becton Dickinson and Company), an antimicrobial supplement. BACTEC drug susceptibility testing was also done for the two primary TB-drugs, streptomycin and ethambutol at concentrations of 6mg ml^{-1} and 7.5mg ml^{-1} respectively, against the H37Rv strain.

A homogenised culture (0.1ml) of all the strains of *M. tuberculosis*, yielding $1x10^4$ to $1x10^5$ colony forming units ml^{-1} (CFU ml^{-1}), were inoculated in the vials containing the plant extract, compound as well as in the control vials (Heifets *et al*. 1985). Two plant extract-free vials were used as controls: one vial was inoculated in the same way as the vials containing the compound [1], and the other was inoculated with a 1:100 dilution of the inoculum (1:100 control) to produce an initial concentration representing 1% of the bacterial population ($1x10^2$ to $1x10^3$ CFU ml^{-1}) found in the vials containing crude extract and the compound. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound that inhibited more than 99% of the bacterial population.

When mycobacteria grow in 7H12 medium containing ^{14}C-labeled substrate, they utilise the substrate and $^{14}CO_2$ is produced. The amount of $^{14}C0_2$ detected reflects the rate and amount of growth occurring in the vial, and is expressed in terms of the Growth Index (GI). Inoculated bottles were incubated at 38°C and each bottle was assayed every day to detect GI, at about the same hour until cumulative results were interpretable. The difference in the GI values of the last two days is designated as DGI. The GI reading of the vials containing the plant extract was compared with the control vial, containing a 1:100 dilution of the inoculum. Readings were taken until the control vials containing a 100 times lower dilution of the inoculum, than the vials with plant extract, reached a GI value of 30 or more. If the DGI value of the vial containing the plant extract was less than the control, the population was recorded to be susceptible to the extract.

Results and Discussion

The plate method

Good growth of *M. tuberculosis* (H37Rv) was evident in the bottles containing only Middlebrook medium, within 5 to 6 weeks. All the results were reported after 6 weeks. The MIC of acetone extract was found to be 0.5mg ml^{-1} for a drug-sensitive strain of *M. tuberculosis*. The acetone extract inhibited growth of the organisms at concentrations ranging from 0.5 to 5.0mg ml^{-1}. The organism was found to be partially susceptible to the water extract at the highest concentration 5.0mg ml^{-1} tested.

The radiometric method

Results were interpreted on day 6 or 7 when the control vials containing the 1:100 dilution of the inoculum reached a GI value of 30 or more (Table 1). The DGI values of the vials containing streptomycin and ethambutol was found to be 4 ±

Table 1: Effect of crude acetone extract of *H. caespititium* and caespitate isolated from it on the growth of the drug-sensitive strain (H37Rv) and drug-resistant strains of *Mycobacterium tuberculosis* by the radiometric method

M. tuberculosis strains and degree of resistance to antibiotic drugs[a]	Lab ref. no. of strains	MIC (mg ml^{-1})	ΔGI[b] values	ΔDGI values of the control vials[b]
Drug-sensitive strain (H37Rv) (crude extract)	ATCC27294	0.1	5.33 ± 2.12	25 ± 4
Drug-sensitive strain (H37Rv) (caespitate)	ATCC27294	0.1	7.33 ± 4.93	25 ± 4
Resistant to I and R	CCK028469V	0.1	7 ± 2	26 ± 3.2
Resistant to S, I and E1	C9	0.1	3 ± 1.73	17.33 ± 3.05
Resistant to S, I, R and E1	C84	0.1	8.66 ± 1.52	23 ± 3.5
Resistant to I, S, R, T1 and C	CGT1296429	0.1	8.3 ± 2.88	23.3 ± 3.51
Resistant to I, R, E2, T1, T2 and O	CCK070370H	0.1	10 ± 3.60	27 ± 5.56
Resistant to I, S, E1, E2, K, R, and T1	CGT1330497	0.1	10.3 ± 2.52	26.33 ± 7.09

[a]I, isoniazid; R, rifampin; S, streptomycin; E1, ethambutol; E2, ethionamide; T1, thiacetazone; T2, terizidone; C, cyclocerine; O, ofloxacin; K, kanamycin. [b]ΔGI values are means ± s.d.

1.41 and 6 ± 3.12 respectively. Crude acetone extract of *H. caespititium* showed inhibitory activity against the sensitive organism at a concentration of 0.1mg ml^{-1}. Among other *Helichrysum* species investigated earlier for their anti-TB activity, it was found that MICs of crude acetone extracts of *H. melanacme* and *H. odoratissimum* against the sensitive strain of *M. tuberculosis* were 0.1 and 0.5mg ml^{-1}respectively (Lall and Meyer 1999). A previous evaluation of the antimicrobial activity of the crude acetone extract of *H. caespititium* showed inhibitory activity against fungi, Gram-positive as well as Gram-negative bacterial species at concentrations ranging from 0.01–1.0mg ml^{-1} (Mathekga and Meyer 2001).

Chemical investigations of *Helichrysum* species have revealed that they are rich sources of acetophenones, flavonoids, sesquiterpenoids and phloroglucinols which are probably used in chemical defence mechanisms of plants against bacteria and fungi (Hilliard 1983). In the present study, the MIC of the phloroglucinol, caespitate, isolated from the plant was found to be 0.1mg ml^{-1} for all the *M. tuberculosis* strains. An earlier antimicrobial investigation of this compound showed it to be highly active against bacteria and fungi, exhibiting an MIC ranging from 0.5µg ml^{-1} to 1.0µg ml^{-1} (Mathekga *et al*. 2000). In another study, caespitin, a phloroglucinol structurally similar to caespitate, also isolated from *H. caespititium,* demonstrated significant antibacterial properties (Dekker *et al.* 1983).

It is generally agreed that at least one acidic hydroxyl group and a certain degree of lipophilicity are required for biological activity of compounds (Tomas-Barberan *et al*. 1990). In the present study, the anti-TB compound isolated from *H. caespititium* bears 3 hydroxyls (phenolic hydroxyls) and lipophilicity (3'-isobutyrylphenyl and but-2-enyl acetate residues). However, it requires further verification by testing structurally related phloroglucinols to determine the essential active regions necessary for significant antituberculosis activity.

The results obtained from the radiometric method were satisfactory and comparable with that obtained from the plate method. The time period required by the radiometric method for susceptibility testing was only 6 to 7 days as compared to 6 weeks by the plate method. Results can therefore be obtained significantly faster than with the plate method because in liquid medium there is more cells to drug contact and due to the shorter incubation time, also less likelihood of breakdown of the tested compounds.

This study provides a probable scientific explanation for the therapeutic potency attributed to *H. caespititium*, claimed by traditional healers in the Free State province of South Africa. The traditional use of *H. caespititium* extract against sores, colds, and tuberculosis could possibly be attributed to the activity of caespitate. The crude acetone extract and the isolated compound, caespitate did not show activity at the level of the two positive controls tested. Although this makes it unlikely that the compound and crude extract themselves are candidates for further development, they could act as templates for analogues and it would be interesting to determine if any synergistic effects might occur if the active compound and established anti-tubercular compounds were administered together. Secondly, it is possible that there could be other antimycobacterial compounds present in the plant as the MIC of the crude extract was similar to the MIC of the isolated compound. Research is underway to isolate and identify other compounds with antimycobacterial activity from the shoots of *H. caespititium*.

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